Challenge Appressoria of *Erysiphe graminis* Fail to Breach Preformed Papillae of a Compatible Barley Cultivar

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ABSTRACT

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To investigate the potential role of papillae in resisting fungal ingress, we tested the ability of *Erysiphe graminis* f. sp. *hordei* to form haustoria in host cells of a compatible barley cultivar at sites of preformed papillae. Oversize, preformed papillae were obtained by incubating inoculated coleoptiles on a $Ca(H_2PO_4)_2$ solution from 9–24 hr after inoculation. These papillae had a heterogeneous ultrastructural appearance which differed substantially from that of normal papillae in barley coleoptiles. After the induction of preformed papillae, the fungus was removed from the epidermis, and a second (challenge) inoculum was applied. In two separate experiments, challenge appressoria located over papilla-free host wall areas had a normal percentage of haustoria, 72 or 52%; those over preformed papillae on the same two sets of coleoptiles had only 24 or 0%, respectively. The preformed papillae appeared to permit few or none of the appressoria located over them to form haustoria, depending on the experimental design. Appressoria located near, but not over, preformed papillae had an intermediate level of haustorium formation that may have been caused by substances diffusing from the papillae or by the papilla margins that were, presumably, too thin to be visualized by interference-contrast light microscopy. The results suggested that preformed papillae can prevent appressoria from producing haustoria in cells of a compatible host plant.

Additional key words: penetration, resistance, wall appositions, cytology, host-pathogen interactions.

A living plant cell commonly reacts to localized injury by forming a cytoplasmic aggregate and depositing a lump of heterogeneous material, termed a wall apposition (6), into the paramural space at the injury site. Such appositions may serve to seal the wound (1) and thus prolong the life of the cell.

Wall appositions (termed papillae) which are incited by fungi, long have been suspected of preventing fungal ingress in certain instances (1), and recent experimental work has begun to clarify this relationship. Aist and co-workers (3,23) presented experimental evidence suggesting that the papillae normally produced by kohlrabi and barley cells do not affect penetration by their respective compatible parasites, *Olpidium brassicae* and *Erysiphe graminis* f. sp. *hordei*. In contrast, the results of other studies suggested that, under appropriate experimental conditions, wall appositions of these plants have the potential to prevent penetrations. Aist (2) showed that penetration of compatible kohlrabi root hairs by *O. brassicae* occurred much less frequently from zoospores encysted on wall sites over mechanically induced wall appositions than from those over apposition-free sites. Waterman et al (23) restricted papilla formation to one end of compatible barley coleoptile cells by centrifugation and obtained evidence that a host cytoplasmic response, such as enhanced papilla formation, can prevent some penetrations by *E. graminis*. Moreover, resistance in incompatible plant-fungus combinations may involve papilla formation. Vance and Sherwood (20,21) used cycloheximide treatments and sequential inoculations to generate experimental correlations between papilla formation and resistance of the nonhost reed canarygrass to pathogenic fungi. Thus, the results of experimental studies on the function of papillae in resisting penetration depend heavily on the conditions of the experiments and on the nature of the host-parasite combinations used, and they should not be assumed to be transferable to other conditions or combinations.

We often have wondered why cytoplasmic aggregates in barley coleoptile cells will persist for 2–3 hr before depositing a papilla (4,7), yet, once deposition begins, the papilla is formed rapidly, within 15–30 min (7). In many cases, the cytoplasmic aggregate apparently does not even deposit a papilla. It is as though two signals are required for papilla deposition: the first calling for the redirection and focusing of cyclosis that result in formation of the cytoplasmic aggregate; the second calling for fusion of vesicles in the aggregate with the plasmalemma, resulting in deposition of the vesicle contents into the paramural space. If this concept is valid, then it should be possible to enhance papilla formation by an appropriately timed administration of fusogenic agents. Calcium ions supplied as chloride or phosphate salts generally have been found to promote membrane fusion in animal systems (10,15,19,22,25), and there is evidence that calcium ions are required for the membrane fusions resulting in plant cell wall formation (5,13).

We recently discovered (authors, *unpublished*) that when barley coleoptiles that have been inoculated with conidia of *E. graminis* f. sp. *hordei* are incubated on a solution of $Ca(H_2PO_4)_2$, oversize papillae are induced and only ~6% of mature appressoria produce haustoria. However, this reduction in percent haustoria is not caused by the oversize papillae; when we repeated the experiment starting with a heat shock that prevented papilla formation during penetration, still only 6% of mature appressoria formed haustoria. Apparently, $Ca(H_2PO_4)_2$ treatment not only enhances papilla formation, but it also directly inhibits further development of appressoria. We then recognized an opportunity to perform an experiment similar to the one cited above involving mechanically induced wall appositions (2). In the present study, we tested the ability of appressoria of *E. graminis* f. sp. *hordei* to penetrate compatible barley cells at sites where preformed, oversize papillae had been experimentally produced. The aim was to obtain further evidence concerning the potential role of papillae in resisting fungal ingress.

MATERIALS AND METHODS

General. *Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal (race B7; the former designation of our culture as race AO [4] was less

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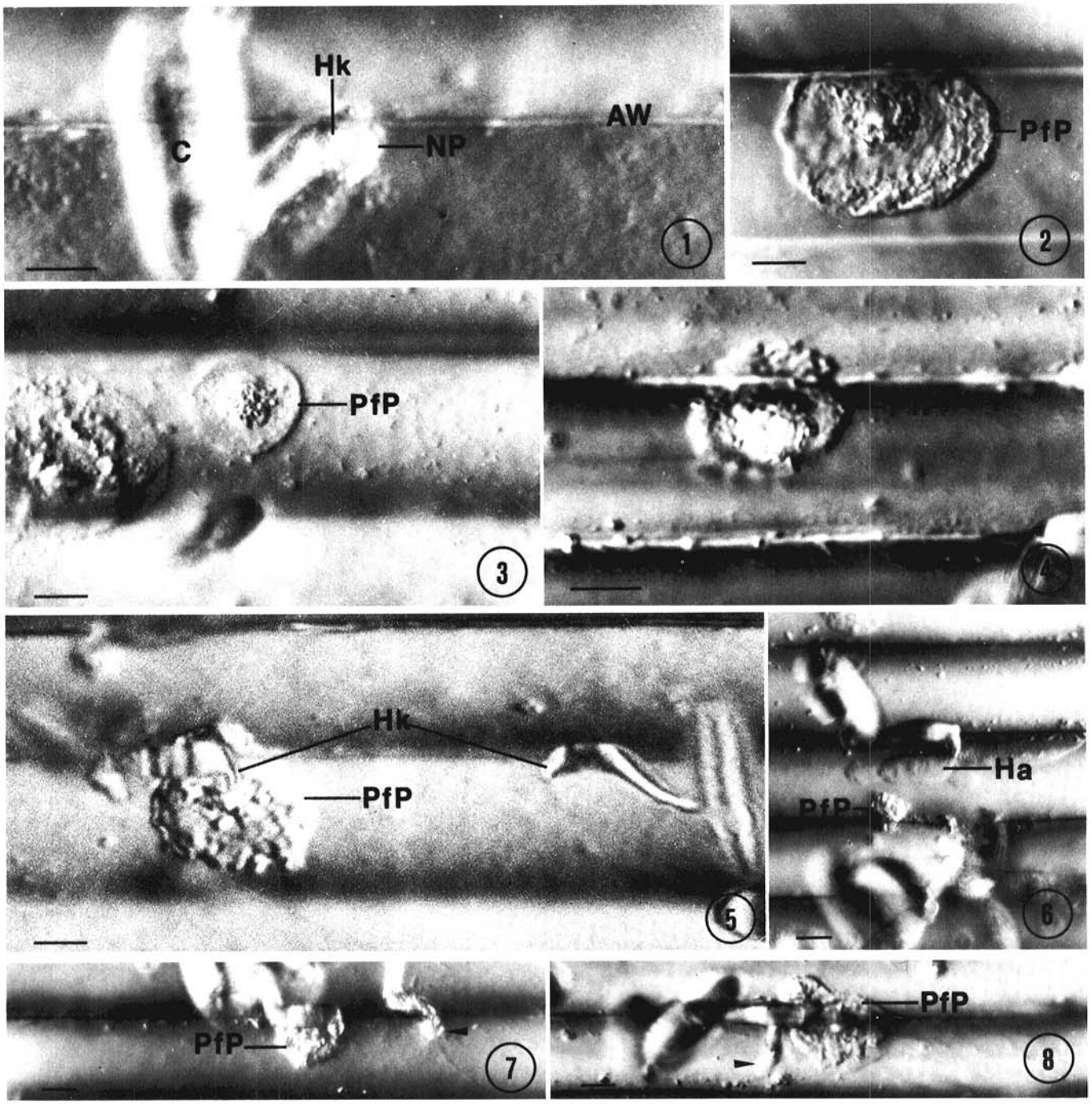
precise) was maintained on compatible barley, *Hordeum vulgare* L. 'Proctor', as previously described (4). Coleoptiles were excised from barley seedlings 7 days after seeding and inoculated with freshly harvested conidia.

Experimental protocols. Data were taken on appressorial groups (pairs or triplets) from the same coleoptiles to minimize effects not attributable to papillae. Each group consisted of one appressorium with its hook located over a preformed papilla (Figs. 5,7,8) and one or two appressoria at designated distances from any preformed papilla (Figs. 5–7). Oversize papillae (Figs. 2–8) were required to provide adequate target areas for challenge appressoria to attack. We used a compatible barley cultivar to ensure a high penetration efficiency over papilla-free wall areas and thus to have a potential for large differences in the data. In this parasite-host system, 50–70% of mature appressoria normally form haustoria, and papillae are formed beneath ~85% of test appressoria (3,4,23). Penetration efficiency (%) (PE) is calculated by the formula: (no. haustoria/no. appressoria) × 100. The protocols described in



Figs. 1-8. Interference-contrast light micrographs of barley (*Hordeum vulgare*) coleoptile epidermal cells inoculated with conidia of *Erysiphe graminis* f. sp. *hordei*. **1,** A normal papilla induced by a challenge appressorium (protocol B). **Figs. 2–4** illustrate different types of preformed papillae produced by protocols A (Fig. 3) and B (Figs. 2, 4). Inducing appressoria were wiped off as described in the text. **5,** A pair of challenge appressoria as they appeared during the selection procedure, before penetration was attempted. The preformed papilla was produced by protocol A. **6,** A challenge appressorium has penetrated and developed a haustorium about 25 μm from a preformed papilla (protocol B). **7,** Two appressoria that failed to penetrate, one over a preformed papilla and another that was initially over a papilla-free wall region but later induced a normal papilla (arrowhead). Note the striking difference in the sizes of these two papillae. **8,** This appressorium first failed to breach the preformed papilla from its primary hook, then produced a secondary hook over a papilla-free host wall area, breached the wall and formed a haustorium (arrowhead). Abbreviations: AW = anticlinal wall; C = conidium; Ha = haustorium; Hk = primary hook of appressorium; NP = normal papilla; PfP preformed papilla. Calibration bars = 10 μm.

Tables 1 and 2 were used.

Light microscopy. For the PE data in Table 4, coleoptiles were placed on glass slides over a drop of 0.01 M $Ca(NO_3)_2$ and viewed with Zeiss interference-contrast optics at ×500. Photographs were made of each encounter site included in the data in Tables 3 and 4 and were used to relocate selected sites and/or confirm the accuracy of the data.

Electron microscopy. Normal papillae were obtained by first incubating dissected coleoptiles in Plexiglas mounts (4) on 0.01 M $Ca(NO_3)_2$ for ~ 6 hr, then inoculating them by dusting on freshly collected conidia with an artist's brush, and finally incubating them for another 22 hr. Preformed papillae were obtained by protocol B (Table 2) and fixed 12 hr after challenge inoculation. Papilla formation was complete at fixation.

Fixation was accomplished at room temperature by gradually increasing the concentration of NaOH-PIPES (14)-buffered (0.08 M, pH 8.0) glutaraldehyde (5%) added dropwise over 1 hr to the 0.01 M $Ca(NO_3)_2$ incubation medium. Final fixative concentrations were thus ~20% of those indicated above. Next, the coleoptiles were fixed 1 hr in full-strength glutaraldehyde-buffer, and repeatedly rinsed with 0.2 M NaOH-PIPES (pH 6.8). Further processing involved post fixation in 2% OsO_4 in NaOH-PIPES buffer (0.18 M, pH 6.8) for 1 hr, dehydration in an acetone series, immersion in propylene oxide for 1.5 hr, infiltration with Spurr's medium (16) with propylene oxide as the solvent, and polymerization at 70 C for 16 hr.

Encounter sites were selected for thin sectioning from embedded specimens viewed directly before and during trimming or from 25 μm-thick sections that were cut transversely to the plane of the coleoptiles, mounted in immersion oil on glass slides, and scanned by light microscopy. Serial thin-sections were collected on Formvar- and carbon-coated 1 × 2-mm slot grids, stained with methanolic uranyl acetate followed by lead citrate, and examined in a Philips EM-200 electron microscope.

TABLE 1. Protocol A for the induction of preformed papillae in barley coleoptiles and challenging them with a second inoculation of *Erysiphe graminis* conidia

Procedural steps	Duration (hr)	Elapsed time (hr)
1. Dissect and mount coleoptiles between Plexiglas wafers as previously described (4).	1	1
2. Incubate on 0.01 M $Ca(NO_3)_2$ at 18 C in the dark; then inoculate by dusting-on conidia with a brush.	6	7
3. Incubate on 0.01 M $C(NO_3)_2$ as in 2 (above).	10	17
4. Incubate on a 0.01 M $Ca(H_2PO_4)_2$ solution adjusted to pH 5.8–6.0 with $Ca(OH)_2$ (a white precipitate forms).[a]	8	25
5. Rinse underside with 0.01 M $Ca(NO_3)_2$ and brush aside germinated conidia with a probe made from a human eyelash.	1	26
6. Incubate on fresh 0.01 M $Ca(NO_3)_2$.	5	31
7. Heat shock the coleoptiles (3)[b]; then challenge-inoculate by dusting-on conidia as before.	1	32
8. Incubate on fresh $Ca(NO_3)_2$; then record data on and photograph selected[c] encounter sites.	18	50

[a] This step is critical to the development of oversize papillae formed in response to the inducing appressoria.

[b] Heat shock inhibits new papilla formation in response to challenge appressoria. Moreover, without heat shock, challenge appressoria in this protocol have a penetration efficiency of only ~8%, even over papilla-free wall regions.

[c] Only turgid appressoria with well-formed hooks (4) were selected, one to five appressorial pairs per coleoptile.

RESULTS

Preformed papillae produced by both protocols A and B (Figs. 2–8) differed from normal papillae (Fig. 1) in several respects at the light microscopic level. First, they were much larger than normal papillae (Fig. 7). Second, preformed papillae typically were composed of two zones, an inner, more refractile zone and an outer, coarsely granular zone. Normal papillae usually appeared homogeneously refractile. Third, preformed papillae varied in appearance: some had a central core surrounded by a granular, noncontiguous, narrow ring (Fig. 3); others had a central core surrounded by a contiguous, wide ring (Figs. 4,7); and still others were composed almost entirely of the outer, granular ring (Figs. 2,5,8). Normal papillae usually differed from one another only in diameter. Protocol B more consistently yielded preformed papillae of the types shown in Figs. 4, 5, 7, and 8 than did protocol A, and these types were selected for the experimental and ultrastructural studies because they were most numerous and appeared to be more uniformly thick than the others.

In preliminary ultrastructural comparisons, both normal and preformed papillae were composed of interspersed areas of various textures and staining intensities (Figs. 9–12), which is typical of wall appositions (6). Relative to normal papillae (Figs. 9, 10), however, preformed papillae (Figs. 11, 12) were broader, had a much higher proportion of darkly staining materials and were irregularly laminated. Thus far, cell wall areas subtended by preformed papillae have been distinctly thicker than both those subtended by normal papillae and those not associated with papillae.

Challenge appressoria penetrated host cells through wall areas subtended by preformed papillae produced by protocol A much less frequently than through papillae-free wall areas (Table 3). This threefold difference (24 vs 72%) in penetration efficiency (PE) was highly significant by χ^2 analysis ($P > .005$). Forty-four percent

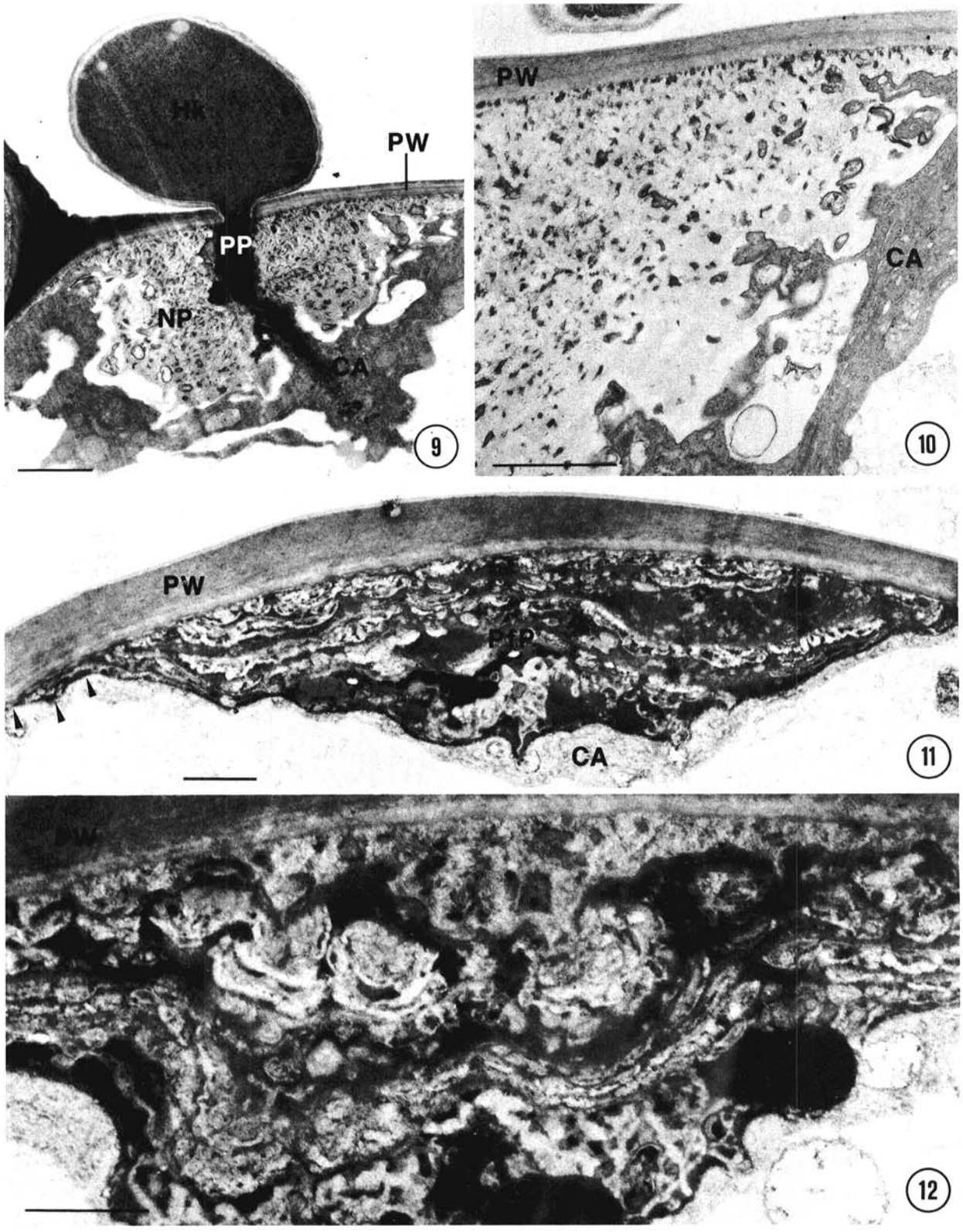
TABLE 2. Protocol B for the induction of preformed papillae in barley coleoptiles and challenging them with a second inoculation of *Erysiphe graminis* conidia[a]

Procedural steps	Duration (hr)	Elapsed time (hr)
1. Prepare coleoptiles as described by Takamatsu et al (18) and float them on 0.01M $Ca(NO_3)_2$ in petri dishes; then inoculate in a spore settling tower.	1	1
2. Incubate on 0.01 M $Ca(NO_3)_2$ at 18 C in the dark.	9	10
3. Incubate on a 1:1 (v/v) mixture of 0.01 M $Ca(NO_3)_2$ and 0.01 M sodium phosphate buffer, pH 7.5.[b]	15	25
4. Incubate on 0.01 M $Ca(NO_3)_2$ and wipe off germinated conidia with a moist cotton swab; challenge inoculate.	1	26
5. Incubate on 0.01 M $Ca(NO_3)_2$; then select and photograph appropriate appressoria.[c]	12	38
6. Determine penetration efficiency of selected appressoria and rephotograph.	12	50

[a] Separate, but concurrent, controls were incubated for 25 hr, then inoculated and incubated for another 24 hr before data were taken.

[b] Critical to the consistent formation of oversize papillae.

[c] Selection was as in protocol A, (Table 1) except that two or three appressorial triplets were chosen per coleoptile.



Figs. 9-12. Transmission electron micrographs that compare and contrast the ultrastructure of full-sized, normal (Figs. 9–10), and preformed (Figs. 11–12) papillae in inner epidermal cells of barley (*Hordeum vulgare*) coleoptiles at encounter sites with *Erysiphe graminis*. **9,** A median section through the zone of host cell penetration showing portions of the appressorial hook and penetration peg of the parasite and the periclinal wall, a normal papilla, and the cytoplasmic aggregate of the host cell. **10,** Another section through the same papilla (Fig. 9) that details its internal structure at higher magnification. Note the numerous membrane profiles within the homogeneous central matrix of the papilla and its convoluted margin composed of matrix, membrane profiles, and enclaves and embayments of the cytoplasmic aggregate. **11,** A median section (cf Fig. 9) that shows the thickened periclinal host wall, a preformed papilla, and the sparse cytoplasmic aggregate of the host cell. Note also the breadth of the papilla, its irregular laminations, and its thin, tapered border (arrowheads). **12,** Another section of the same papilla (Fig. 11; cf Fig. 10) that depicts at higher magnification the varied structure and staining intensity typical of these oversize papillae. Complete serial sectioning of this encounter site revealed that the host cell was neither penetrated nor was its periclinal wall even partially breached by the inducing parasite. Abbreviations: CA = host cytoplasmic aggregate; Hk = primary hook of appressorium; NP = normal papilla; PfP = performed papilla; PP = penetration peg; PW = periclinal wall. Calibration bars = 1 μm.

TABLE 3. Relationships between penetration efficiencies (PE) and locations of appressorial hooks of *Erysiphe graminis* f. sp. *hordei* with respect to preformed papillae (PfP) of *Hordeum vulgare* produced according to protocol A (Table 1)

	Locations of appressorial hooks	
	over PfP	> 20 μm from PfP
(No. haustoria)/ (No. appressoria)	6/25	18/25
PE	24%	72%

of appressoria located over these preformed papillae developed penetration pegs from their primary hooks. In this experiment, nearly all host cells had produced preformed papillae.

The experiment then was repeated, but this time protocol B was used and separate controls were prepared and evaluated concurrently. In this case a similar, but even greater, effect was associated with preformed papillae (Table 4). No significant difference (χ^2 analysis, $P = 0.5$) in PE (~50%) occurred between challenge appressoria located on control coleoptiles and those >50 μm from preformed papillae on treated coleoptiles (Fig. 4), whereas none of the challenge appressoria located over preformed papillae (Figs. 7,8) penetrated from their primary hooks (PE = 0%). Challenge appressoria located within 10 μm of, but not over, preformed papillae had an intermediate PE (24%) that was significantly different ($P > .005$) from all other PE's. Three appressoria with primary hooks that failed to penetrate through preformed papillae subsequently penetrated host cells from secondary hooks that developed over adjacent, papilla-free wall sites (Fig. 8). In this experiment, at least 84% of the primary hooks of appressoria ≤10 μm from preformed papillae and at least 78% of those of appressoria >50 μm from preformed papillae attempted penetration of host cells that had produced preformed papillae.

DISCUSSION

The marked reduction in the PE of challenge appressoria associated with preformed papillae can be variously interpreted. It may have been a direct effect of the preformed papillae. Alternatively, the inducing appressoria may have directly altered their penetration sites, rendering them unsuitable for penetration by the challenge appressoria. These alterations could include structural modification (swelling) of the host wall or leakage of toxic metabolic fungal by-products resulting from inhibition of the appressoria by $Ca(H_2PO_4)_2$. For technical reasons, we were unable to execute an experiment designed to evaluate such possible effects of the inducing appressoria. The reduction in penetration efficiency could not have been caused by a cell-wide resistance elicited by the inducing inoculum (12), since the vast majority of primary appressorial hooks not located over preformed papillae attempted penetration of host cells that also had produced preformed papillae.

The effects on challenge appressoria associated with preformed papillae often were not immediately lethal, since many penetration pegs and secondary hooks were formed. Moreover, the effects were localized in a zone corresponding to host wall areas subtended by the preformed papillae and their immediate surroundings.

The intermediate penetration efficiency (24%) observed for appressorial hooks over the zone adjacent to preformed papillae (Table 4) could be an effect of the thin, peripheral papillar material, perhaps seen only by electron microscopy (Fig. 11), or of a hypothetical toxin that could diffuse from its site of deposition in the papilla. Preformed papillae produced by protocol A also permitted an intermediate penetration efficiency, indicating that they were less effective in preventing ingress than were those produced by protocol B. A range of effectiveness is not surprising in view of the differences between protocols A and B (cf. Tables 1 and 2) and the apparent inability of normal papillae to prevent ingress (3,4,23).

Our results, even though equivocal, provide further experimental evidence that wall appositions have the potential to prevent fungal ingress, thus supporting inferences drawn from earlier experimental studies (2,20,21,23). Correlated chemical, physical, and ultrastructural characterizations may suggest why appressoria are able to penetrate host cells through normal papillae (4) more readily than through preformed ones.

TABLE 4. Relationships among penetration efficiencies (PE) and locations of appressorial hooks of *Erysiphe graminis* f. sp. *hordei* with respect to preformed papillae (PfP) of *Hordeum vulgare* produced according to protocol B (Table 2)

	Locations of appressorial hooks			
	over PfP	≤ 10 μm from PfP	> 50 μm from PfP	on control coleoptiles
(No. haustoria)/ (No. appressoria)	0/50	12/50	206/400	200/400
PE	0%	24%	52%	50%

Since compatible hosts can be made to form wall appositions that apparently are quite effective in preventing ingress (2,23, and the present results), one might expect that under more natural conditions an incompatible plant may exclude a fungal pathogen by forming appositions. Possible examples of such interactions may be found in studies of nonhost plants inoculated with plant pathogenic fungi (8,9,11,17,20,24). Critical experimental work is needed to link such resistance more specifically to papilla formation.

The effects of $Ca(H_2PO_4)_2$ solutions—inhibition of appressorial germination and enhanced papilla size—are poorly understood. An elaborate series of experiments with various calcium salts at pH increments between 6.0 and 8.0 would help clarify the underlying mechanisms and further indicate the degree of similarity between enhanced papilla formation and calcium-mediated secretion in animal cells.

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